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A Comparison of Four Immunometric Assays for Myeloperoxidase Using Luminescent and Colorimetric Signal Detection

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Summary: This communication presents four different assay systems for the determination of myeloperoxidase in body fluids. One is based on conventional chemiluminescence, two on luminescence-amplified enzyme measurement using either spiroadamantane-1,2 dioxetanes with alkaline phosphatase or luminol/peroxidase/4-iodophenol coupled with a peroxidase label.

The assays covered the range 0–600 µg/l peroxidase. Established reference range for EDTA plasma from healthy volunteers gave an upper limit of 250 µg/l (95% confidence limits). Intra-assay coefficients of variation were less than 5% for all assays, as seen in compound precision profiles. Inter-assay variation was less than 10% throughout the whole concentration range.

Kinetic curves for the light production were performed over a 2 hour period for the enhanced luminescence assays. Dynamic ranges of these assays were compared with the conventional colorimetric assay using 4-nitrophenyl phosphate – alkaline phosphatase.

The assay was standardized using a commercially available myeloperoxidase preparation with defined enzymatic activity. The protein content was estimated and the laboratory standard compared and calibrated in mass units.

Introduction

Non-radioisotopically labelled immunoassays are becoming increasingly important, especially those which are simple and robust, and attain the lower detection limits of the radioimmunoassay or immunoradiometric assay. The results of different variants of immunometric assays with luminescent and colorimetric detection are presented for the detection of myeloperoxidase in body fluids. Myeloperoxidase is used as a marker in diseases with granulocyte involvement (1–3) or in myeloid cell disorders (4–5), and its measurement complements the assays for other granulocyte components, namely lactoferrin (6, 7) and elastase-α_1-proteinase inhibitor complexes (7) developed and published from this laboratory (7). Although all three components usually correlate well with each other (8), they are secreted from different sources (2, 3) and may therefore be used to differentiate between various cellular reactions during pulmonary insult, for example, during sepsis, protozoan, bacterial or viral attack (7, 9, 10).

The assays described here are either with "direct labelling" where anti-myeloperoxidase has been labelled with a luminogen, or by "universal labelling" of the same antibody with an amidocaproylbiotin-N-hydroxysuccinimide ester. In the latter case, the detection system consisted of streptavidin-labelled peroxidase or alkaline phosphatase together with a colorimetric or luminogenic substrate (11–13).

The luminogen used for direct coupling was 9-[N-(4 aminobutyl)-N-ethyl]-aminobenzo(f)phthalazine-1,4 (2H,3H) dione (ABEN). The luminescent substrate for alkaline phosphatase was 3-[(2′-spiroadaman-

tane)-4-methoxy-4-(3″phosphoryloxy)phenyl]-1,2 dioxetane (AMPPD), and the substrate for peroxidase was luminol/peroxide with 4-iodophenol as enhancer.

An alkaline phosphatase-labelled enzyme immunoassay using 4-nitrophenyl phosphate as substrate was performed for comparison.

The assays were tested under routine conditions, which included the establishment of a reference range for EDTA plasma from healthy volunteers (7), together with a comparison between myeloperoxidase, lactoferrin and elastase- α_1-proteinase inhibitor complexes in cases of bronchial infection.

The luminescence-enhanced enzyme immunoassays and the immunoenzymometric assay with colorimetric measurement were based on microtitre plate technology (14), whereas the chemiluminescent immunometric assays used polystyrene balls as solid phase (15).

Materials and Methods

Materials

Myeloperoxidase was obtained as reference material from Behring-Calbiochem. (Marburg a. d. L., FRG) and was declared as 98% pure giving a single band after electrophoresis.

The myeloperoxidase used as standard in the assays was derived from a pathological urine sample containing approximately 125 mg/l myeloperoxidase. Dilutions were carried out in foetal calf serum (Gibco, Paisley, GB).

Streptavidin was purchased from Boehringer Mannheim (Mannheim, FRG) or from Biogenzia Lemania (Bochum, FRG).

Streptavidin-peroxidase, anti-myeloperoxidase and streptavidin-alkaline phosphatase were bought from DAKO (Hamburg, FRG).

AMPPD was purchased from Tropix Inc. (Bedford, MA. USA) as a 10 g/l solution.

ABEN was synthesised in the laboratories of the clinical research unit of this hospital.

The luminol/peroxide and enhancer were commercial reagents from Amersham International (Amerlite Signal Reagent), Braunschweig FRG. Diethanolamine buffer (Art. Nr. 14000) and 4-nitrophenyl phosphate (Art. Nr. 14001) were obtained in ready to use form from Merck, Darmstadt, FRG.

Polystyrene balls (6.4 mm diameter) were purchased from Spherotech Kugeln GmbH, Fulda, FRG.

Microtitre plates were obtained from Greiner, Nürtingen, FRG, and were γ-radiated. Both clear (Cat. No. 756061) and white opaque plates (special lot — Cat. No. 701648) were used.

Other buffer substances were obtained from different sources. Luminometers for reading 75 × 12 mm tubes/cuvettes were from Lab. Prof. Dr. Berthold (Type LB 952-16 T; 250-sample chain-luminometer), Wildbad, FRG, or from Stratec (Type SL-300; 300-sample rack-luminometer), Birkenfeld, FRG.

The microtitre plate luminometer (Luminoskan) was from ICN-Flow Laboratories (Labsystems Oy), Helsinki, SF.

All machines were capable of performing data reduction with integrated or connected microprocessors/desk top computers.

Methods

Immunometric assays were used throughout. Table 1 shows the flow scheme for the assays based on polystyrene balls, table 2 for assays based on microtitre plates. The basic details of the assays have been published several times for different components elsewhere (7, 12) and need not be further dealt with here.

Tab. 1. Assay flow scheme for polystyrene ball-based immunometric assays for myeloperoxidase using "direct-labelling" of the antibody.

20 µl sample or standard 200 µl ABEN-labelled anti myeloperoxidase (rabbit) 1 anti-myeloperoxidase coated ball (rabbit)
Incubate 45 min at ambient temperature on horizontal rotator (170 min^{-1}). Wash with 2 × 5 ml demineralized water.
Transfer balls to measuring cuvettes (Sarstedt 12 × 75 mm — No. 55.476).
Add 300 µl catalase (Boehringer Mannheim Cat. Nr. 106836 — 1:200 dilution in 0.15 mol/l NaCl, 0.015 mol/l NaN_3, pH 5).
Load luminometer and initiate light reaction with alkaline peroxide (1 Perhydrit tablet (Merck Art. Nr. 7201) in 200 ml 0.33 mol/l NaOH).
Integrate signal over 2 s and perform data reduction.

Tab. 2. Assay flow diagram for microtitre plate-based immunoassays for myeloperoxidase using "indirect" labelling of the antibody with amidocaproylbiotin

20 µl sample or standard 200 µl biotinylated anti myeloperoxidase (rabbit) 1 anti-myeloperoxidase coated well (rabbit)
Incubate 45 min at 18—25 °C on microtitre plate shaker. Wash with 4 × 250 µl 0.4 ml/l Triton X-405 in demineralized water.
200 µl Streptavidin-labelled alkaline phosphatase for AMPPD and 4-nitrophenyl phosphate as substrate (dilution 1 : 30 000)
or
200 µl Streptavidin-labelled peroxidase for luminol-peroxide 4-iodophenol as substrate (dilution 1 : 20 000)
Incubate 30 minutes at 18—25 °C on microtitre plate shaker. Wash as above.
200 µl AMPPD 0.4 mmol/l in 0.1 mol/l diethanolamine buffer (Merck Art. Nr 14000 — ready to use), pH 9.8*
or
200 µl Amerlite signal reagent (contents not declared), pH 9.0*
or
200 µl 4-nitrophenyl phosphate (Merck Art. Nr. 14001) — 1 tablet in 15 ml 0.1 mol/l diethanolamine buffer, pH 9.8*
For the luminescence measurement, load Luminoskan luminometer and scan plate (0.1 s/well) at time(s) required.
For the colorimetric determination stop reaction after 10 min with 50 µl 0.25 mol/l EDTA-Na_4/NaOH, pH 13 and read in microtitre plate colorimeter (Biorad EIA reader 2550 or 3550) at 405 nm.

* The substrate incubations were all carried out at ambient temperature (18—25 °C)

The assay buffer and throughout, unless otherwise specified, was: 0.05 mol/l phosphate, 0.15 mol/l NaCl, 0.5 ml/l Triton X-405, pH 7.5. The substrates for the (luminescent-enhanced) enzyme immuno-assays were used as follows:

a) AMPPD – 0.4 mmol/l in 0.1 mol/l diethanolamine buffer

b) 4-nitrophenyl phosphate – 1 tablet in 15 ml diethanolamine buffer.

c) Luminol-peroxidase Signal Reagent Buffer (contents not declared), pH 9.0

All substrate working solutions were prepared directly before use and were discarded afterwards. Only AMPPD was stored as a stock solution in the dark at 4–7 °C.

Statistics

Non-parametric statistics were used throughout, after the *Kolmogorov-Smirnoff* test showed non-normal distributions in several data groups ($\alpha > 0.05$). Correlations were made using the *Spearman* rank test, graphical representations using the two regression line technique – $y = a + bx$ and $x = a + by$. The relevant percentiles and median values were used as locators.

Results and Discussion

Results

a) Polystyrene ball-based assays using ABEN as label

Figure 1 shows a typical standard curve for assays using a direct label.

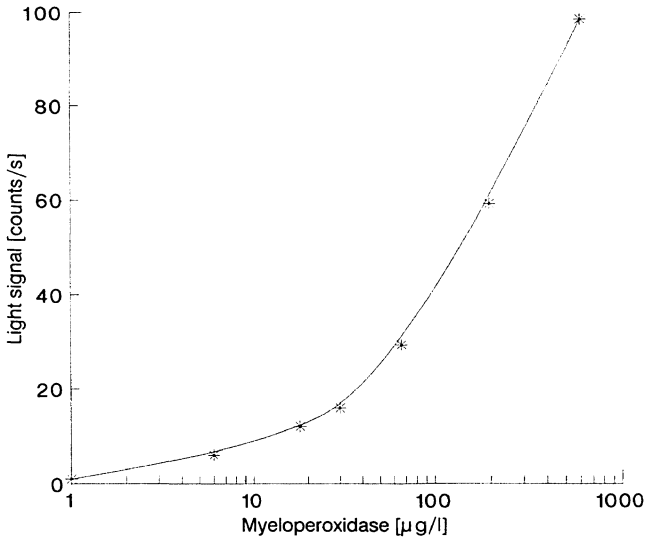

Fig. 1. Standard curve (1-step assay) for myeloperoxidase using ABEN-labelled anti-myeloperoxidase as the liquid phase antibody. Assay details as in table 1. Reference range for fresh EDTA plasma from healthy volunteers up to 250 µg/l. The counts on the ordinate are arbitrary and cannot be compared directly with those from the microtitre plate luminometer.

b) Establishment of a reference range for plasma from normal healthy volunteers

EDTA plasma was used throughout. The time between blood sampling and separation of plasma was 2–30 min (7). Haemolysed samples were discarded, as were plasma samples from volunteers with elevated aminotransferases, γ-glutamyl transferase, C-reactive protein and neopterin. Table 3 shows 10 consecutive assays using the assay described in table 1.

Tab. 3. Distribution of myeloperoxidase concentrations in 182 healthy volunteers and in 49 patients with pulmonary infections.

Percentiles	Myeloperoxidase (µg/l)	
	Healthy volunteers	Pulmonary infection
2.5	82	238
16	107	351
50 (Median)	137	680
84	192	1230
97.5	245	2430
Range of values	78–308	238–11290

Upper limit of the reference range for healthy volunteers in fresh EDTA plasma was 250 µg/l (95% confidence limits)

c) Comparison of white blood cell constituents

Elastase-α_1-proteinase inhibitor, lactoferrin and myeloperoxidase values in 49 patients with pulmonary disease or infection using the assay in table 1 were compared. The correlation between elastase-α_1-proteinase inhibitor and lactoferrin was 0.667, with myeloperoxidase 0.725. The correlation between lactoferrin and myeloperoxidase was 0.521. All correlation coefficients were significant ($p < 0.001$ – *Spearman* rank correlation). The distribution of myeloperoxidase concentrations is given in table 3.

d) Inter- and intra-assay variations

Table 4 shows the basic data of the assays described in section *a)* above in the form of compound precision profiles for intra-assay variation, and conventionally with mean values and coefficients of variation for inter-assay comparison.

Tab. 4. Precision data for the polystyrene ball-based assay.

a. Compound precision profile
(data from duplicate determinations)

Range (µg/l)	No. of sample pairs	Mean CV (%)
<200	428	4.27
200–600	416	4.03

b. Inter-assay variation
(Data from 20 consecutive assays)

Control serum	Mean concentration (µg/l)	Mean CV (%)
Control LF-1	140	8.22
LF-2	398	9.90
LF-3	243	6.38

e) Microtitre plate-based luminescence enhanced immunoenzymometric assays (LEIA) using an alkaline phosphatase label

The luminescent substrate AMPPD is stable at pH 9.8. The substance is a dioxetane, which is a direct precursor of molecules which luminesce, independent of bio- or chemiluminescence. Through the action of alkaline phosphatase, the $AMPPD^-$-anion yields a constant light signal (11). Figure 2 shows the light reaction kinetics in the form of a standard curve read at 1, 5, 10, 60, 90 and 180 minutes after substrate was pipetted into the microtitre plate wells. The light signal could be measured over 60 minutes without appreciable loss of signal or loss in precision or dynamic range of the assay.

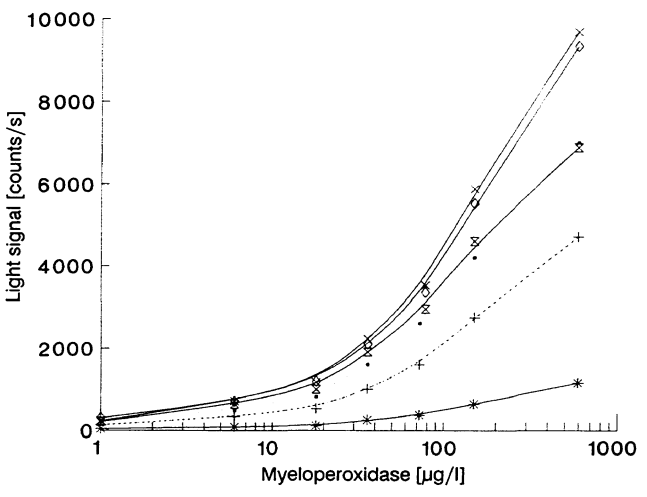

Fig. 2. Light kinetic curves over the first 3 hours for the AMPPD-alkaline phosphatase system. Concentration of AMPPD 0.4 mmol/l, pH 9.8. The counts on the ordinate are arbitrary.
— ✳ — 1 min ---+--- 5 min ····■···· 10 min
— × — 30 min — ◇ — 60 min —⧖— 180 min

f) Conventional immunoenzymometric assay using alkaline phosphatase 4-nitrophenyl phosphate

As a control system, 4-nitrophenyl phosphate in the colorimetric determination replaced the AMPPD (see (e)). The colour reaction was stopped after 10 minutes with 50 µl of 0.25 mol/l EDTA adjusted to pH 13 with solid NaOH. The colour was read at 405 nm. Colourless microtitre plates replaced the opaque ones used in (e) above.

g) Peroxidase system using luminol/peroxide 4-iodophenol

The combination of peroxidase with luminol and peroxide has been known for over a decade (13) and is used with success commercially. The light signal can be "enhanced" (amplified) by using phenolic derivatives such as 4-iodophenol.

The light signal is also stable as shown in figure 3. The assay can be measured up to 30 minutes after substrate addition. The reagents used here were exactly as supplied by Amersham International in their Amerlite Signal Reagent kit.

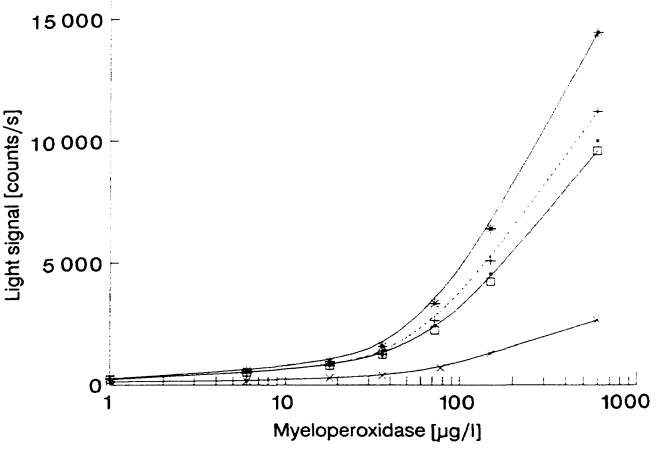

Fig. 3. Light kinetic curves for the peroxidase- luminol/peroxide system over a 2 hour time period with a reaction pH of 9.0.
— ✳ — 1 min ---+--- 10 min ····■···· 20 min
—□— 30 min — × — 120 min

h) Dynamic ranges of microtitre plate assays

Figure 4 shows standard curves for the two luminescence-enhanced enzyme immunoassays as well as for the assay with colorimetric detection. All three assays were set up at the same time using identical reagents, with the exception of the streptavidin-enzyme/substrate reagents. The dynamic range of the assays, measured by the ratio of the signal of the 600 µg/l standard to the zero standard (after substraction

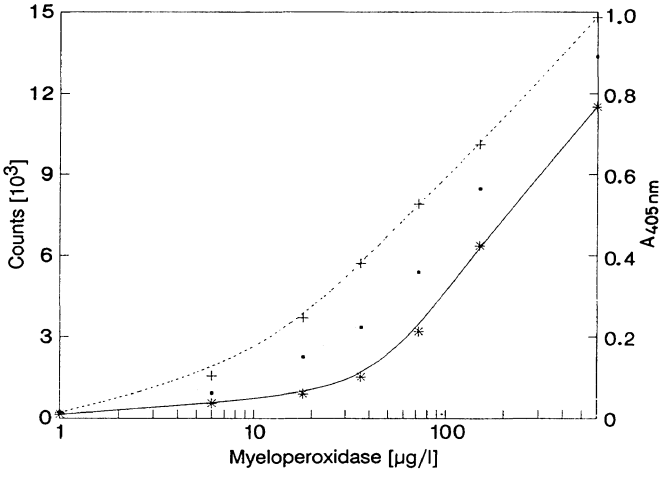

Fig. 4. Comparison of standard curves from AMPPD, luminol/peroxide and 4-nitrophenyl phosphate as signal reagents. All assays were set up identically on one microtitre plate. Only the substrates differed (see table 2). All reactions took place at ambient temperature (18–25 °C).
— ✳ — LEIA-POD --+-- LEIA-AP ····■···· EIA-AP

of the reagent blank) was 180 : 1 for the luminol-peroxidase system, 150 : 1 for AMPPD and 65 : 1 for the 4-nitrophenyl phosphate system. These data were the optimal values measured 5 minutes after substrate addition for the luminol/peroxide system, after 30 minutes for the AMPPD system and after 10 minutes for the 4-nitrophenyl phosphate system.

i) Precision of microtitre plate assays

The precision of microtitre plate assays is similar to that for the polystyrene ball-based assays. This is clearly shown by comparison of the data in tables 4 and 5.

Tab. 5. Precision data from the microtitre plate assay using AMPPD – alkaline phosphatase as label

a. Compound precision profile
(Data from duplicate determinations)

Range (µg/l)	No. of sample pairs	Mean CV (%)
<200	337	4.02
200–600	223	4.17

b. Inter-assay variation
(Data from 20 consecutive assays)

Control serum	Mean concentration (µg/l)	Mean CV (%)
Control LF-1	134	7.48
LF-2	402	9.41
LF-3	223	7.14

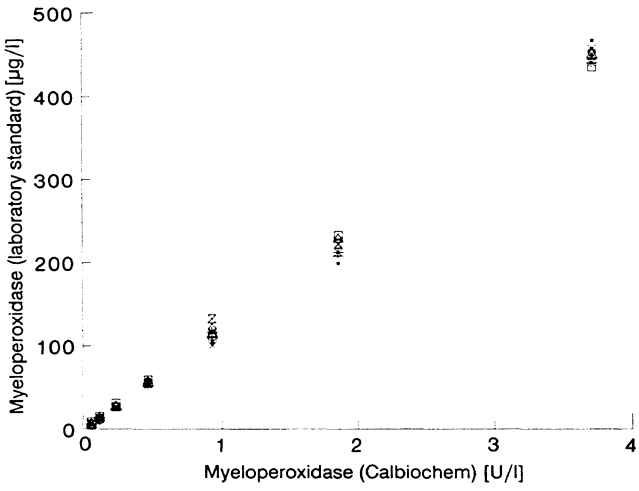

Fig. 5. Relationship between commercial myeloperoxidase preparation (abscissa) and laboratory internal standard in pathological urine (ordinate). Each point is composed of results from 8 individual assays. The immunological reactivity of both preparations is the same over a wide measuring range. The antibody reacts specifically, as the urine also contains elastase complexes (110 mg/l) and lactoferrin (85 mg/l).
▪ Assay 1 + Assay 2 ✳ Assay 3 □ Assay 4
× Assay 5 ◇ Assay 6 △ Assay 7 x Assay 8
Regression r = 0.999
Intercept = 0 Slope = 120

Only irradiated microtitre plates were suitable, the capacity of normal plates to adsorb antibody not being sufficient. Normal plates could be made into "high-capacity" plates by pretreatment with poly phenylalanine-lysine/pentan-1,5-dial as described in the literature (15, 16).

j) Standardisation of the myeoloperoxidase assay

As there are no international reference preparations for myeloperoxidase, comparison was made between the laboratory standard, extracted from urine of a patient with acute renal rejection, and a commercially available myeloperoxidase preparation (Behring-Calbiochem) with declared activity and purity.

The protein content of the commercial preparation was determined using bovine serum albumin as standard and the Biorad protein assay based on Coomassie blue reaction. A value of 120 µg/U was measured. Figure 5 shows serial dilutions of the commercial standard from 8 assays as measured against the laboratory internal urinary standard. The correlation data show that an excellent consensus between both standards was achieved. Mass units were used in routine assays as an immunoassay does not necessarily reflect enzymic activity.

k) Comparison of results from the direct labelled assay using polystyrene balls with those from the AMPPD/alkaline phosphatase microtitre plate assay

One hundred and sixty plasma samples were measured simultaneously in 8 consecutive assays using ABEN or AMPPD as luminogen. Myeloperoxidase concentrations ranged from 72 to 1310 µg/l. The *Spearman* rank correlation coefficient was 0.984, the slopes of the regression lines $y = a + bx$ and $x = a + by$, being 0.972 and 1.018 respectively. This data confirmed that both assays measured the same concentrations within statistically acceptable limits.

l) Final discussion points

We demonstrate not only new assay systems for the non-radioisotopic determination of myeloperoxidase in body fluids, but also support the claims made over a decade ago (17) that if an assay is well designed, the choice of label is often of secondary importance.

The assays based on enhanced luminescence allow one of the basic criticisms and drawbacks of chemiluminescence-based measurements – namely the "one-off" measurement – to be overcome.

The use of a luminescent detection system in combination with an enzyme label allows the *Beer-Lambert* law for colorimetric measurements to be overcome. The low, non-specific signal of AMPPD reduces the lower detection limit, at the same time increasing the measuring range of the assay.

The stability of reagents is excellent, for example the streptavidin-alkaline phosphatase used in this study was kept in solution for over 2 years stored at 4 °C. ABEN-labelled antibodies have shelf lives of over 5 years, when stored under −27 °C.

The use of microtitre plate luminometers, combined with assays which need no reagent injection step in the instrument, make this system attractive and reduces the costs of instrumentation. It also allows those who aready possess such a luminometer, for example, the Amerlite system, to develop and implement their own assays, either using peroxidase or alkaline phosphatase as labels.

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